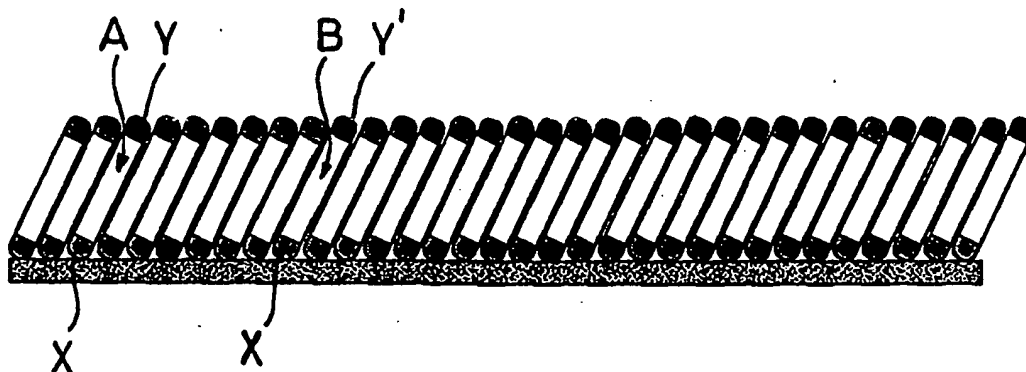




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<b>(21) International Application Number:</b> PCT/SE95/00736 <b>(22) International Filing Date:</b> 16 June 1995 (16.06.95) <b>(30) Priority Data:</b> 9402116-9 16 June 1994 (16.06.94) SE <b>(71) Applicant (for all designated States except US):</b> PHARMACIA BIOSENSOR AB [SE/SE]; S-751 82 Uppsala (SE). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> LIEDBERG, Bo [SE/SE]; Sofielundsvägen 7, S-585 97 Linköping (SE). TENGVALL, Pentti [SE/SE]; Blåklintsgatan 4, S-582 46 Linköping (SE). <b>(74) Agents:</b> WIDÉN, Björn et al.; Pharmacia AB, Patent Dept., S- 751 82 Uppsala (SE).	<b>(81) Designated States:</b> CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i>	

**(54) Title:** A METHOD OF PREPARING A MOLECULAR GRADIENT ON A SOLID SUBSTRATE SURFACE, A SOLID SUBSTRATE WITH A MOLECULAR GRADIENT AND USE OF THE SUBSTRATE

**(57) Abstract**

The invention relates to a method of preparing a continuous molecular concentration gradient along at least part of a solid substrate surface, which gradient provides for gradually changing surface properties in at least one surface dimension. The gradient is prepared by applying a diffusion matrix (2) to the substrate surface (1) and cross-diffusing at least two different components, selected from molecules and mixtures of molecules capable of binding to the substrate surface, towards each other through the matrix. The invention also relates to a solid substrate with such a gradient as well as to the use of the substrate for studying molecular adsorption patterns.

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A METHOD OF PREPARING A MOLECULAR GRADIENT ON A SOLID  
SUBSTRATE SURFACE, A SOLID SUBSTRATE WITH A MOLECULAR  
GRADIENT AND USE OF THE SUBSTRATE

5           The present invention relates to molecular  
interactions with solid substrate surfaces, and more  
particularly to the preparation of improved chemical  
(molecular) gradients on solid substrates for the study of  
molecular interactions as well as a solid substrate  
10 surface having such a gradient or gradients.

          Studies of interaction phenomena of biopolymers, such  
as proteins, at solid surfaces have been performed for  
various purposes. Exemplary are the behaviour of the  
adsorption and desorption of blood proteins or the  
15 adhesion and proliferation of different types of mammalian  
cells on polymeric materials. Conventionally, several  
surface preparations with varied but constant chemical  
properties have been used, e.g. based upon the binding of  
one or more chemical species to the surface. In recent  
20 years, however, single surfaces where one chemical  
property of the surface is continuously varied, or in  
other words "gradient surfaces", have been prepared. With  
such surfaces the effects of a continuum of selected and  
controlled physical-chemical properties can be studied in  
25 one experiment on the surface.

          Such chemical gradients have previously been  
generated on Si/SiO<sub>2</sub> wafers (1-8; the figures referring to  
a list of references at the end of the description) and on  
polymers (9-11). Common to all these gradients is that  
30 they utilize the substrate surface as one of the  
components in the gradient. For example, the work by  
Elwing et al. (1-5, 8) is based on the silane coupling  
chemistry and the Si/SiO<sub>2</sub> system, and all of their work  
focuses on wettability gradients where the hydrophobic  
35 part consists of immobilized dichloro-dimethylsilane  
molecules and the hydrophilic part of the Si/SiO<sub>2</sub>  
substrate itself. The preparation of wettability gradients  
on polymer surfaces by Pitt (9) and Lee et al. (10-11)

utilizes a scanning radio frequency (RF) discharge- or a scanning corona discharge apparatus, respectively. Pitt utilizes a cover connected to a scanning apparatus which allows him to vary the exposure time of the RF plasma along the surface. The corona discharge (10-11) is generated via a knife edge. By increasing the power of the discharge during the translation of the knife-edge along the surface they were able to control the oxidation of the outermost surface and thereby generate a wettability gradient.

The present invention relates to a novel and improved method of preparing chemical, or molecular, gradients on solid substrates, which method does not rely on using the substrate surface as one of the components but utilizes at least two different molecules in the gradient. It is readily seen that this will heavily increase the number of possible chemical combinations in the gradient.

In accordance with the method of the invention, such a continuous mixed molecular gradient, which provides for gradually changing surface properties in at least one surface dimension, is prepared along at least part of a solid substrate surface by applying a diffusion matrix to the substrate surface and cross-diffusing at least two different components, selected from molecules and mixtures of molecules capable of binding to the substrate surface, towards each other through the matrix. As the cross-diffusing molecules diffuse towards each other in the diffusion matrix, they are successively bound to the surface. When the two diffusion "flows" meet, the diffusing molecules will compete for the binding sites at the surface and a monolayer exhibiting a concentration gradient in respect of the diffusing molecules will result.

The term "cross-diffusing" is to be interpreted in a broad sense. Usually, two different components, i.e. two different molecules, or molecule mixtures, or one molecule and one molecule mixture, are cross-diffused towards each other from opposed parts of the diffusion matrix to

provide a linear gradient in one surface dimension. It is also conceivable, however, that two additional components may cross-diffuse towards each other transversely to the first-mentioned diffusing components to provide for gradually changing surface properties in a second substrate surface dimension. In this case, the four different components will diffuse from a respective side of a square or rectangle. More complex gradient patterns may be obtained by diffusion of components from the sides of a triangle, pentangle, hexangle or other polygons.

The cross-diffusing molecules or molecule mixtures are suitably provided in supplies arranged in such contact with the diffusion matrix that diffusion of the respective molecules into the diffusion matrix is permitted. While these molecule supplies may be arranged in arbitrary locations on a substrate surface, they are preferably arranged at end parts of a substrate surface. If desired, each supply may be in diffusive contact with two or more separate substrate surface members such that a molecular concentration gradient may simultaneously be prepared on each substrate surface member.

The supplies for the diffusing molecules may be of various types. Usually, the supplies contain the respective molecule or molecule mixtures dissolved in a solvent. In a simple embodiment each supply is a cavity made in a solid-like diffusion matrix, which cavity contains the molecule solution. The supply may, for example, also be a container having a semi-permeable wall or membrane through which the molecule or molecules may diffuse. In a currently preferred embodiment, however, the supplies are open-pore solid bodies which contain a solution of the respective molecule or molecules. The pore size should preferably be selected to provide for a diffusion rate within the pores that is higher than that in the diffusion matrix. Examples of suitable such open-pore solid bodies are glass filters and cellulose filters.

The diffusion matrix should provide for a controlled diffusion of the molecules. Low-viscosity fluids will give

a too rapid diffusion and uncontrolled diffusion, whereas the diffusion rate in a solid diffusion matrix, such as a polymer like silicone, would be too slow. Diffusion matrices providing the desired diffusion conditions for the molecules may be selected from viscous fluids, such as polyethylene glycol, glycerol, silicone oil, etc; gels, preferably organic gels, such as polysaccharide gels; and solid/liquid mixtures of chromatographic bed type, such as silica particles/liquid. An example of a suitable gel is cross-linked dextran, such as Sephadex® (Pharmacia Biotech AB, Uppsala, Sweden).

In case the diffusion matrix is a gel or a solid/liquid mixture, the liquid used for swelling the gel or used in the particle slurry, respectively, may conveniently, but need not, be the same as the solvent for the diffusing molecules in the supplies thereof. The diffusing molecules must in any case, however, be soluble in the diffusion matrix liquid.

The diffusing molecules may be selected to bind to the substrate surface per se or to specific reaction sites on the surface. The substrate may, for example, be a metal, such as gold, silver, copper, aluminium, platinum or palladium; a dielectric material, such as silicon or tin oxide; a semiconductor material, such as gallium arsenide or doped silicon; or a polymer. The binding of the molecules to the substrate surface is preferably covalent or at least covalent-like.

The desired chemical properties of the bound molecular concentration gradient may be provided directly by the bound diffusing molecules. Alternatively, they may be introduced afterwards by an additional reaction step where the bound molecule is reacted with an appropriate agent, as will be described in more detail below.

The diffusing molecules or molecule mixtures are preferably of organic nature. They may, for example, be macromolecules, such as proteins or polypeptides, e.g. monoclonal antibodies, or nucleic acids.

A suitable type of molecules has the schematic general formula X-R-Y, wherein X represents one or more anchoring groups, R is one or more, especially 1-3, straight or branched hydrocarbon chains, optionally interrupted by one or more hetero atoms (selected from oxygen, nitrogen or sulfur) and/or unsaturations (selected from double and triple bonds), and Y is a group (of any type, size and shape) which, optionally together with R, either directly or after reaction with an activating agent provides a desired chemical property to the molecule or the modified substrate surface.

The above-mentioned hetero atoms and unsaturations may be situated in any position(s) along the chain as well as in branched parts.

One or more anchoring groups X may be provided at the end of a hydrocarbon chain R, e.g. two groups X being bound to a terminal carbon atom. One or more such hydrocarbon chains may, in turn, be bound to a single group Y. Alternatively, a single hydrocarbon chain bound to a group Y may be branched and support one or more groups X at the end of each branch.

Examples of functional groups Y which may be subjected to further chemical activation are hydroxy, mercapto, dithio, carboxy, amino, cyano, formyl, hydrazyl, carbonyl, epoxy, vinyl and halo (fluoro, chloro, bromo, iodo). A broad range of procedures to activate these groups are available in the literature (18, 19).

In an advantageous group of compounds X-R-Y, R is a straight (non-branched) hydrocarbon chain  $(CH_2)_n$ , wherein n preferably is higher than 10. This type of compounds is capable of forming highly organized assemblies with the groups Y pointing to the ambient (12-13; 16-18).

By selecting diffusion pairs with different tail groups Y, for example, the following combinations of chemical characters may be prepared:

hydrophobic/hydrophilic, negative/positive charge, polar/apolar, L-/D-isomers, cis/trans-isomers, acid/base, rigid/flexible molecule tails, chromophoric/non-

chromophoric, fluorophoric/non-fluorophoric, chemiluminescent/non-chemiluminescent and receptor site/non-receptor site.

It is, of course, also possible to prepare gradients by cross-diffusing molecules with different chains R or chain lengths and thereby create gradients with varying tail group mobility.

As mentioned above, the tail groups in the initially prepared gradient can also become activated by subsequent chemical procedures. The outcome of such subsequent activation (organic synthesis) of modified surfaces in general requires that the surface modification agents X-R-Y are strongly bound to the substrate surface via the anchoring group X. Self assembled monolayers based on long chain thiols, sulphides and disulphides have proven very useful for obtaining highly organized and chemically very robust surface modifications on the coinage metals copper, silver and gold (12-15). The  $\omega$ -substituted alkyl thiol-gold system (12-13, 16-18) is particularly attractive for this gradient application because of the strong and specific Au-S bond formation and the high orientational order of the alkyl chains with the tail group pointing towards the ambient (see WO 90/05303). The structural behaviour of these monolayers is also well-known.

In a subgroup of compounds X-R-Y for binding to a substrate surface of especially gold, silver, copper, aluminium, platinum or palladium, the anchoring group X is selected from:

- asymmetrical or symmetrical disulphide (-SSR'Y', -SSRY), sulphide (-SR'Y', -SRY), diselenide (-SeSeR'Y', -SeSeRY, selenide (-SeR'Y', -SeRY),
- nitrile (-CN), isonitrile, nitro (-NO<sub>2</sub>), selenol (-SeH), trivalent phosphorous compounds, iso-thiocyanate, xanthate, thiocarbamate, phosphine, thiophosphates,
- thioacid or dithioacid (-COSH, -CSSH),
- imidazoles, triazoles, carboxylic acid,

wherein R' and Y' are as defined above for R and Y.



The invention also provides a solid substrate surface of the type described above which comprises a molecular concentration gradient of at least two different components selected from molecules and molecule mixtures bound to the substrate surface.

Gradient surfaces in accordance with the invention may be used for various purposes, for example for the study of adsorption patterns of molecules, especially proteins. Such patterns may, for example, conveniently be studied by surface plasmon resonance (SPR) and related evanescent wave based methods.

In the following, the invention will be described in more detail in respect of a specific embodiment of the invention with reference to the accompanying drawings, where:

Fig. 1 is a schematic representation of a prior art non-continuous mixed monolayer pseudo-gradient consisting of discrete surfaces;

Fig. 2 is a schematic representation of a continuous mixed monolayer gradient prepared according to the invention;

Fig. 3 is a schematic top view of an embodiment of a diffusion matrix-substrate glass-filter arrangement for the preparation of mixed molecule concentration gradients;

Fig. 4 is a schematic sectional view of the arrangement in Fig. 3;

Figs. 5a-d show ellipsometric film thicknesses of gradients: a) HS-(CH<sub>2</sub>)<sub>15</sub>-CH<sub>3</sub>/HS-(CH<sub>2</sub>)<sub>16</sub>-OH, b) HS-(CH<sub>2</sub>)<sub>15</sub>-CH<sub>3</sub>/HS-(CH<sub>2</sub>)<sub>16</sub>-CN, c) HS-(CH<sub>2</sub>)<sub>16</sub>-OH/HS-(CH<sub>2</sub>)<sub>16</sub>-CN and d) HS-(CH<sub>2</sub>)<sub>11</sub>-COOH/HS-(CH<sub>2</sub>)<sub>9</sub>-CH<sub>3</sub>, the step size between each measurement point being 0.635 mm; and

Figs. 6A and 6B are stacked XPS core level spectra of a HS-(CH<sub>2</sub>)<sub>15</sub>-CH<sub>3</sub>/HS-(CH<sub>2</sub>)<sub>16</sub>-CN gradient showing a well-defined gradient region with a length of about 4-6 mm.

A prior art non-continuous mixed monolayer pseudo-gradient is schematically shown in Fig. 1 and consists of five planar substrate surface elements S to which mixed monolayers of molecules A and B are bound in varying

mutual proportions. The molecule A has an anchoring part X and a tail part Y, and the molecule B has an anchoring part X and a tail part Y'. As seen from the left to the right over all the substrate surfaces 5 in the figure, the concentration of A gradually decreases, whereas the concentration of B gradually increases. Assume that tail Y has one chemical property, for example, hydrophobic and tail Y' has another chemical property, for example, hydrophilic. Then a non-continuous hydrophobic/hydrophilic "gradient" is obtained with the hydrophilicity increasing to the right in the figure. It is readily seen that it is relatively complicated to prepare such a non-continuous gradient, a number of separate mixtures of molecules A and B having to be bound to a respective surface element.

Fig. 2 illustrates a corresponding continuous gradient  $Af(x)B_{1-f(x)}$  ( $0 < f(x) < 1$ ) along the substrate surface on a single substrate according to the present invention. Such a gradient may conveniently be obtained by the arrangement shown in Figs. 3 and 4.

In Figs. 3 and 4, three separate solid substrates 1 are covered by a matrix 2, for example, of gel type, such as a cross-linked dextran gel. Two glass filters 3 and 4 are arranged at each end of the solid substrates 1. As indicated in Fig. 4, a solution of a first molecule X-R-Y is introduced into glass filter 3, and a solution of a second molecule X-R-Y' is introduced into glass filter 4. In the two molecules, X is an anchoring group capable of binding the molecules to the substrate surfaces, R is a hydrocarbon chain and Y and Y' are tail groups with different chemical properties. The solvent for the respective molecules X-R-Y and X-R-Y' may suitably be the same as the liquid used for swelling the matrix gel 3.

Once the two molecule solutions have been introduced into the respective glass filters 3, 4, the molecules X-R-Y and X-R-Y' start to cross-diffuse towards each other in the matrix gel 2. During the diffusion the molecules will successively bind to the substrate surface 1. In Fig. 4 the molecules have both reached a central region from

either side. On continued diffusion, the two molecules will compete for the binding sites on the surface and a mixed concentration gradient of the two molecules will be obtained. The result will be a continuous gradient of the type shown in Fig. 2.

The invention will now be illustrated further, by way of example only, by the following non-limiting Example.

#### EXAMPLE

##### A. Preparation of gradients based on thiols and gold

###### Gold film preparation:

Gold films  $\approx 2000$  Å thick were prepared onto pre-cut glass strips 40 x 4 x 0.3 mm. The gold-coated strips were stored in a sealed container. They were cleaned in ethanol and then placed on the bottom of a glass petri-dish (Figs. 3 and 4).

###### Preparation of diffusion matrix:

Six grams of Sephadex<sup>®</sup> LH-20 (Pharmacia Biotech AB, Uppsala, Sweden) and 18 grams of ethanol (95 %) were added on top of the gold strips. Excess ethanol was then allowed to evaporate from the petri-dish until a 2-3 mm thick matrix (completely swollen) was formed on top of the gold substrates. Glass filters with a pore size of 100-150  $\mu$ m were placed on top of the matrix 40 mm apart (Fig. 4).

###### Diffusion of reagents in matrix:

Long chain  $\omega$ -substituted alkyl thiols  $\text{HS}-(\text{CH}_2)_n\text{-Y}$  ( $\text{Y} = \text{OH}$ ,  $\text{CN}$  and  $n = 15, 16$ ) and  $\text{HS}-(\text{CD}_2)_{11}\text{-COOH}$  were synthesized according to well-known methods (20). Simple  $n$ -alkyl thiols  $\text{HS}-(\text{CH}_2)_{15}\text{-CH}_3$  and  $\text{HS}-(\text{CH}_2)_9\text{-CH}_3$  were obtained from Fluka and used as received. The thiols were dissolved in ethanol (95 %) to a final concentration of 2 mM. 200  $\mu$ l of each solution were pipetted into the two glass filters with pore size 100-150  $\mu$ m (Figs. 3 and 4). The molecules were then allowed to diffuse towards one another in the matrix. The diffusion front geometry was determined by the shape of the glass filters (Fig. 3). The petri-dish was sealed during the entire diffusion process to prevent evaporation of the solvent. The process was typically interrupted after 48 hours and the so-prepared

gradients were carefully rinsed in 95 % ethanol and distilled water and then ultrasonicated for 5 minutes in 95 % ethanol and distilled water, respectively, in order to ensure that all Sephadex<sup>®</sup> was removed from the surface.

5 The gradients were then stored in 95% ethanol until use.

#### B. Analysis of gradients

Gradients on the gold/glass strips prepared above were analysed by ellipsometry, contact angle measurements and X-ray photoelectron spectroscopy (XPS).

#### 10 Ellipsometry:

Ellipsometric measurement of the thicknesses of HS-(CH<sub>2</sub>)<sub>15</sub>-CH<sub>3</sub>/HS-(CH<sub>2</sub>)<sub>16</sub>-OH, HS-(CH<sub>2</sub>)<sub>15</sub>-CH<sub>3</sub>/HS-(CH<sub>2</sub>)<sub>16</sub>-CN, HS-(CH<sub>2</sub>)<sub>16</sub>-OH/HS-(CH<sub>2</sub>)<sub>16</sub>-CN monolayers prepared according to the procedure described under paragraph A above are

15 summarized in Figs. 5a-c. The plots show that the monolayers consist of highly organized assemblies with a thickness of  $21.5 \pm 1.0$  Å on the polar side (-OH, -CN) and  $20.5 \pm 1.0$  Å on the apolar side (-CH<sub>3</sub>). The observed thicknesses are in good agreement with the geometrical

20 thicknesses determined from space filling models and experimentally obtained tilt angles of the polymethylene chains (15). The observed difference in thickness between the polar and apolar side  $\approx 1$  Å is also consistent with a chain length difference of one methylene unit, 1.5 Å, and

25 with a tilt angle of 25 - 40 ° (15) included in the modelling of the geometrical thickness ( $= 1.5 \cdot \cos(40^\circ) \approx 1.15$  Å). A gradient where the components in the diffusion pair have different chain lengths HS-(CD<sub>2</sub>)<sub>11</sub>-COOH/HS-(CH<sub>2</sub>)<sub>9</sub>-CH<sub>3</sub> was also prepared and the ellipsometric

30 thickness was plotted in Fig. 5d. The gradient region with a length of about 5-6 mm becomes clearly visible in this particular case as the step in layer thickness is  $\approx 5$  Å.

Contact angle measurements: Contact angle data with water for the gradients shown in Fig. 5 are summarized in Table

35 I below.

TABLE I

	Diffusion pair	Q <sub>H2O</sub> a)	
	A/B	A	B
5			
	HS-(CH <sub>2</sub> ) <sub>15</sub> -CH <sub>3</sub> /HS-(CH <sub>2</sub> ) <sub>16</sub> -OH	112±4 °	<10° b)
	HS-(CH <sub>2</sub> ) <sub>15</sub> -CH <sub>3</sub> /HS-(CH <sub>2</sub> ) <sub>16</sub> -CN	112±4°	60±4°
10			
	HS-(CH <sub>2</sub> ) <sub>16</sub> -OH/HS-(CH <sub>2</sub> ) <sub>16</sub> -CN	<10° b)	60±4°
	HS-(CH <sub>2</sub> ) <sub>9</sub> -CH <sub>3</sub> /HS-(CD <sub>2</sub> ) <sub>11</sub> -COOH	105±5°	28±4°

15 a) Contact angles obtained at the very extreme ends of the gradient. A continuous variation in contact angles Q<sub>H2O</sub> between the extreme values are observed in all cases. Observed contact angles with water for single component monolayers with the same chain length: HS-(CH<sub>2</sub>)<sub>15</sub>-CH<sub>3</sub>,  
 20 113-116°; HS-(CH<sub>2</sub>)<sub>9</sub>-CH<sub>3</sub>, 108-112°; HS-(CH<sub>2</sub>)<sub>16</sub>-OH, < 10°; HS-(CH<sub>2</sub>)<sub>16</sub>-CN, 59-62°; HS-(CD<sub>2</sub>)<sub>11</sub>-COOH, 26-30°.

b) Contact angles below 10° are difficult to measure with high accuracy.

25

X-ray photoelectron spectroscopy (XPS):

XPS was used to study the chemical composition along the gradient surface. Figs. 6A and 6B show a series of stacked C(1s) and N(1s) core level spectra of a HS-  
 30 (CH<sub>2</sub>)<sub>15</sub>-CH<sub>3</sub>/HS-(CH<sub>2</sub>)<sub>16</sub>-CN monolayer. The step size is 0.5 mm between each analysis spot (spectrum) and the analysis area is 0.2 mm wide. The peak at 399.5 eV in Fig. 6A is the N(1s) signature from the -CN group. The evolution of the C(1s) peaks is shown in Fig. 6B, where the large peak  
 35 at 285 eV is characteristic for -CH<sub>2</sub>- units within the polymethylene chain. The peak near 287 eV appears after 6 mm from the extreme hydrophobic (CH<sub>3</sub>) end and originates from the nitrile carbon and the methylene carbons close to

the nitrile group ( $-\text{CH}_2-\text{CN}$ ). These plots show furthermore that the length of the gradient is 4-6 mm.

The ellipsometric, contact angle, and XPS data presented above clearly indicate that continuous gradients  
5 were formed on gold with the described cross-diffusion method according to the invention of two or more long chain  $\omega$ -substituted alkyl thiols.

The invention is, of course, not restricted to the  
embodiments described above and specifically shown in the  
10 drawings but many modifications and changes are within the scope of the general inventive concept as defined in the following claims.

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CLAIMS

1. A method of preparing a continuous molecular concentration gradient along at least part of a solid substrate surface, which gradient provides for gradually changing surface properties in at least one surface dimension, characterized by applying a diffusion matrix (2) to the substrate surface (1) and cross-diffusing at least two different components, selected from molecules and mixtures of molecules capable of binding to the substrate surface, towards each other through the matrix.
2. The method of claim 1, characterized in that two components are cross-diffused towards each other from opposed parts of the diffusion matrix (2).
3. The method of claim 1 or 2, characterized in that each said different molecule is provided in a respective supply (3, 4) arranged in such contact with the diffusion matrix (2) that diffusion of the respective molecules into the diffusion matrix is permitted.
4. The method of claim 3, characterized in that each supply (3, 4) contains a solution of the respective molecule or molecule mixture.
5. The method of claim 3 or 4, characterized in that said molecule supplies (3, 4) are arranged at respective end parts of said substrate surface (1).
6. The method of claim 3, 4 or 5, characterized in that each molecule supply (3, 4) is in diffusive contact with two or more separate substrate surface members (1) for the preparation of a molecular gradient on each substrate surface member.
7. The method of any one of claims 1 to 6, characterized in that each said molecular supply is an open-pore solid

body (3, 4) containing a solution of the respective molecule.

8. The method of claim 7, characterized in that said  
5 open-pore solid body (3) is a glass or cellulose filter.

9. The method of any one of claims 1 to 6, characterized  
in that each said molecular supply is a container  
containing a solution of the respective molecule or  
10 molecules, said container having a permeable wall through  
which the molecules can diffuse into the diffusion matrix.

10. The method of any one of claims 1 to 9, characterized  
in that the diffusion matrix (2) is a, preferably organic,  
15 gel or viscous fluid or a particle/liquid mixture.

11. The method of claim 10, characterized in that the  
diffusion matrix (2) is a polysaccharide gel, preferably  
cross-linked dextran.  
20

12. The method of any one of claims 3 to 11,  
characterized in that the diffusion matrix (2) contains  
the same solvent as that of the respective molecule  
solutions in said supplies.  
25

13. The method of any one of claims 1 to 12,  
characterized in that the substrate surface (1) supports  
reactive sites for binding said different diffusing  
molecules to the surface.  
30

14. The method of any one of claims 1 to 12,  
characterized in that the different diffusing molecules  
are capable of binding to the substrate surface material  
per se.  
35

15. The method of claim 14, characterized in that the  
solid substrate surface is selected from a metal,  
preferably gold, silver, copper, aluminium, platinum and

palladium; a dielectric material; a semiconductor material; and polymeric materials.

16. The method of any one of claims 13 to 15,  
5 characterized in that said binding of the diffusing molecules to the substrate surface is covalent or covalent-like.
17. The method of any one of claims 1 to 16,  
10 characterized in that the method comprises reacting one or both of the diffusing molecules when they have bound to the substrate surface to provide a desired chemical property to the bound mixed molecular layer.
18. The method of any one of claims 1 to 17,  
15 characterized in that one or both of said different diffusing molecules are organic molecules which form a monolayer on the substrate surface.
19. The method of claim 18, characterized in that the  
20 organic molecule or molecules have the general formula X-R-Y, wherein X represents one or more groups capable of anchoring the molecule to the substrate surface, R represents at least one straight or branched hydrocarbon  
25 chain which optionally is interrupted by one or more hetero atoms and/or unsaturations, and Y is a group which, optionally together with R, either directly or after reaction with an activating agent, provides a desired chemical property to the modified substrate surface.
20. The method of claim 19, characterized in that R is a  
30 hydrocarbon chain  $(CH_2)_n$ , wherein n preferably is higher than 10.
21. The method of claim 19 or 20, characterized in that  
35 the group Y is a functional group capable of being reacted further with an agent providing a desired property to the bound mixed molecular layer.

22. The method of claim 21, characterized in that the group Y is selected from hydroxy, carboxy, amino, cyano, formyl, hydrazyl, carbonyl, mercapto, dithio, epoxy, vinyl and halogen.

23. The method of any one of claims 19 to 22, characterized in that the two diffusing molecules differ from each other in that they have different groups Y.

24. The method of any one of claims 19 to 23, characterized in that the two diffusing molecules differ from each other in that they have different groups R.

25. The method of any one of claims 19 to 24, characterized in that the substrate surface is selected from gold, copper, silver, aluminium, platinum and palladium, preferably gold, and that the group X is selected from:

- asymmetrical or symmetrical disulphide (-SSR'Y', -SSRY), sulphide (-SR'Y', -SRY), diselenide (-SeSeR'Y', -SeSeRY, selenide (-SeR'Y', -SeRY),
- nitrile (-CN), isonitrile, nitro (-NO<sub>2</sub>), selenol (-SeH), trivalent phosphorous compounds, iso-thiocyanate, xanthate, thiocarbamate, phosphine, thiophosphates,
- thioacid or dithioacid (-COSH, -CSSH),
- imidazoles, triazoles,
- carboxylic acid,

wherein R' and Y' are defined as for R and Y.

26. The method of any one of claims 1 to 25, characterized in that at least one of said diffusing molecules is a protein or a peptide, e.g. a monoclonal antibody, or a nucleic acid.

27. The method of any one of claims 1 to 26, characterized in that it comprises cross-diffusing at least three different components from mutually different

directions to provide for gradually changing surface properties in at least a second substrate surface dimension.

5 28. The method of claim 27, characterized in that said directions are defined by a polygon selected from a triangle, rectangle, pentangle and hexangle.

10 29. The method of any one of claims 1 to 28, characterized in that said molecular gradient is selected from at least one of the following combinations of chemical characters: hydrophobic/hydrophilic, negative/positive charge, polar/apolar, L-/D-isomers, cis/trans-isomers, acid/base, rigid/flexible molecular  
15 tails, chromophoric/non-chromophoric, fluorophoric/non-fluorophoric, and chemiluminescent/non-chemiluminescent.

20 30. A solid substrate with a surface having a molecular concentration gradient thereon, characterized in that said gradient is formed by at least two different components selected from molecules and molecule mixtures bound to the substrate surface.

25 31. The solid substrate according to claim 30, characterized in that said molecules are selected from those defined in any one of claims 18 to 30.

30 32. Use of a solid substrate according to claim 30 or 31 for studying adsorption patterns of molecules, especially by evanescent wave based methods.

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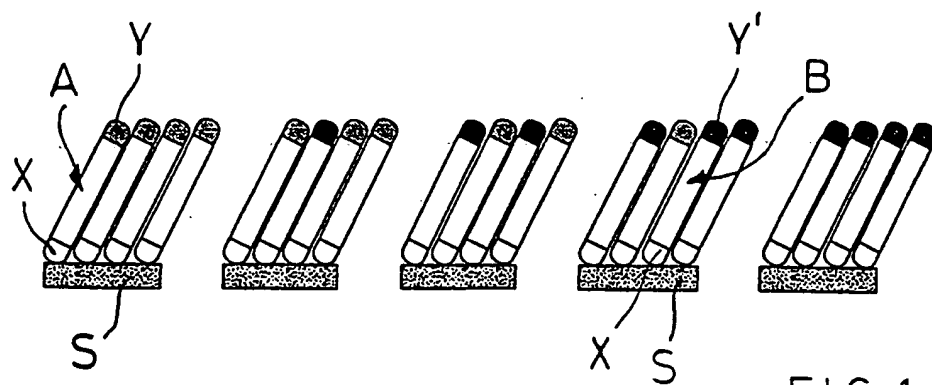


FIG. 1

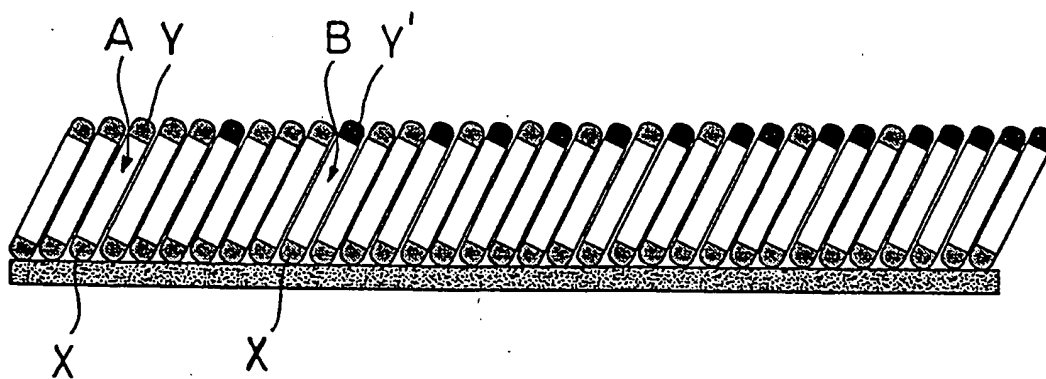


FIG. 2

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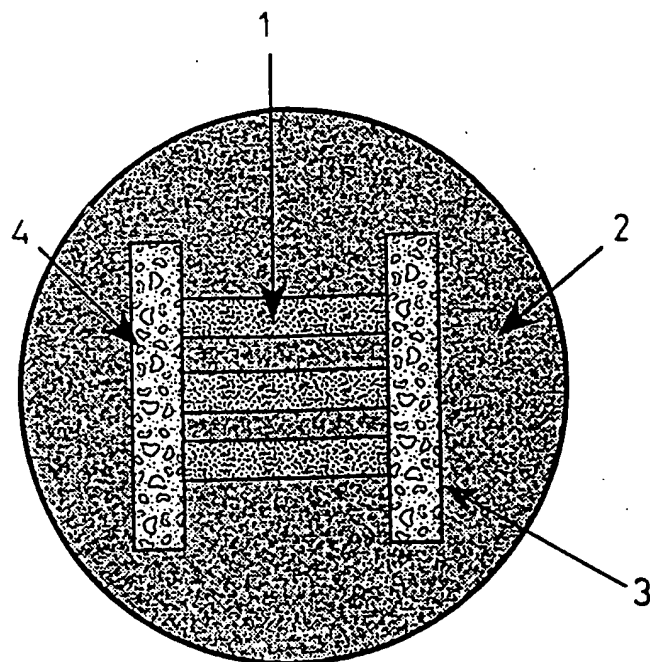


FIG. 3

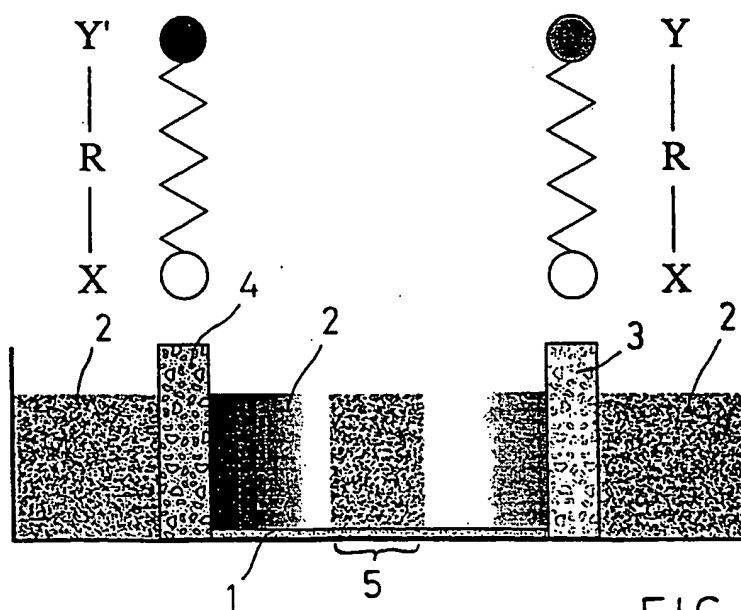


FIG. 4

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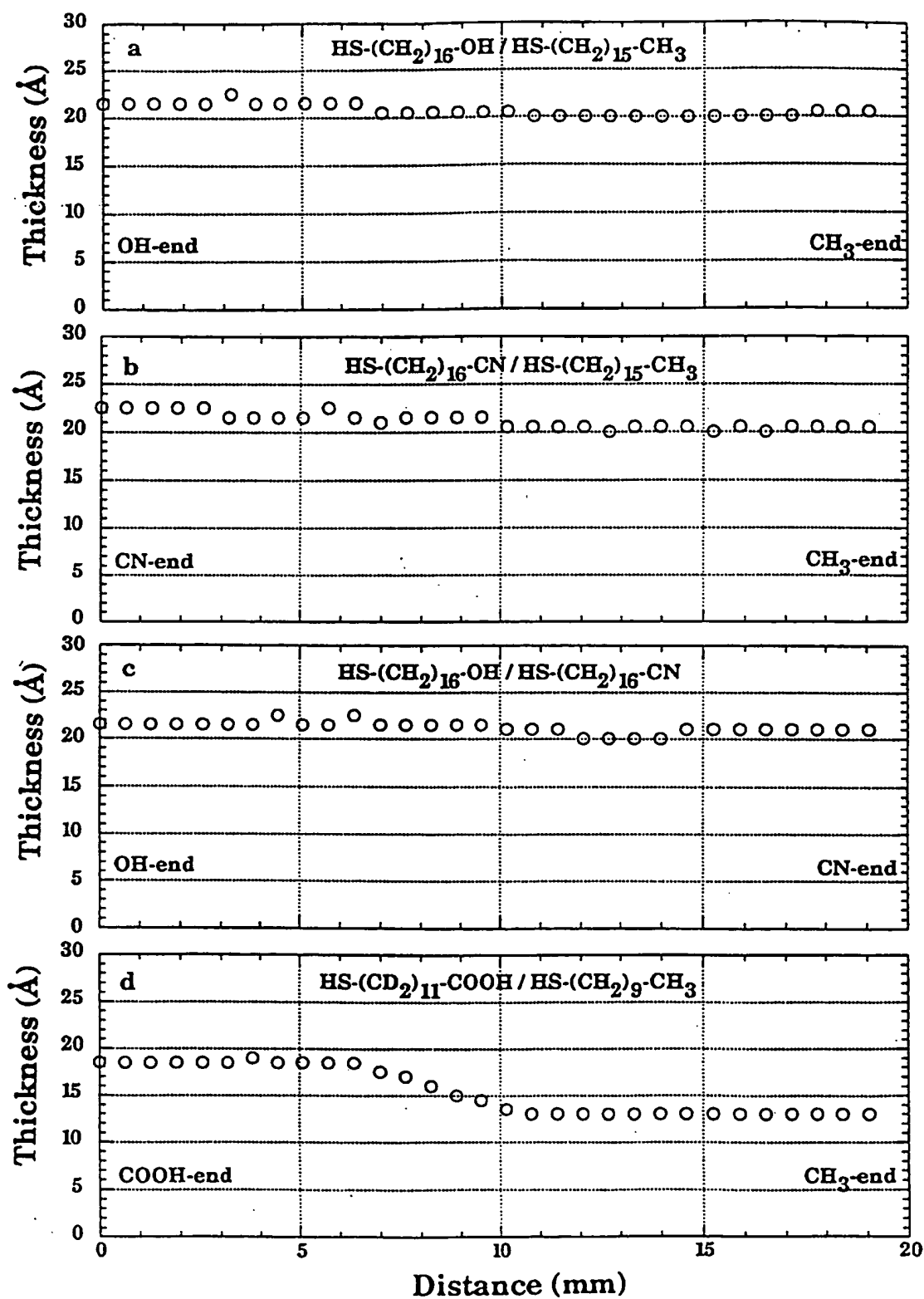


FIG. 5a-5d



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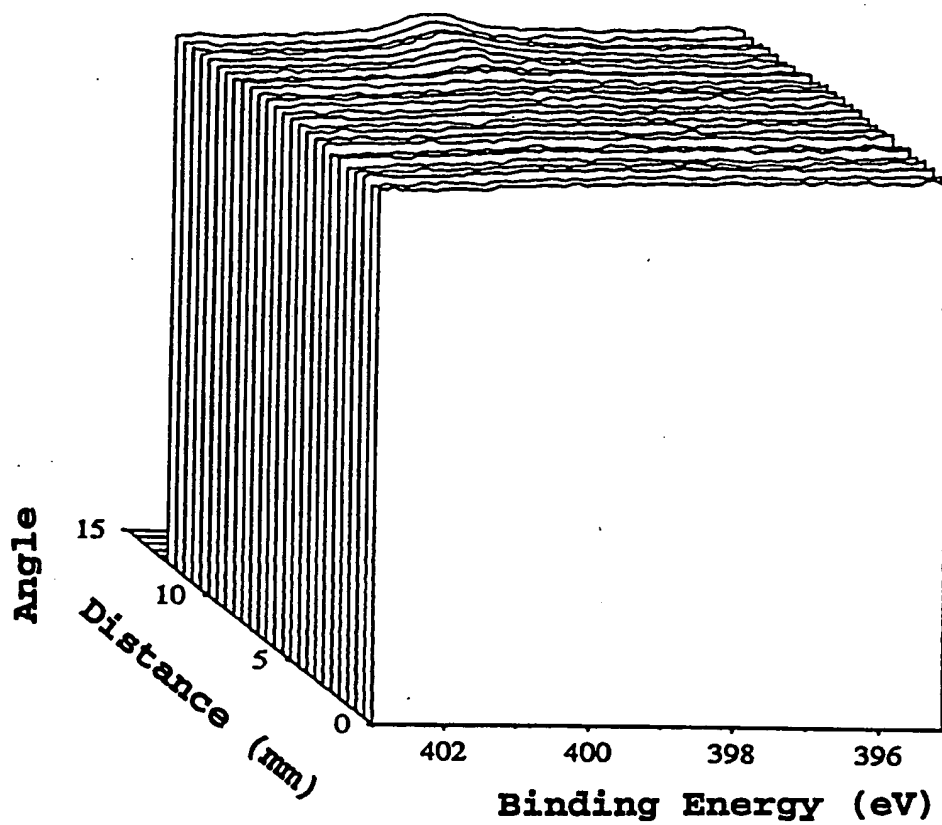
 $-(CH_2)-$ 

FIG. 6A

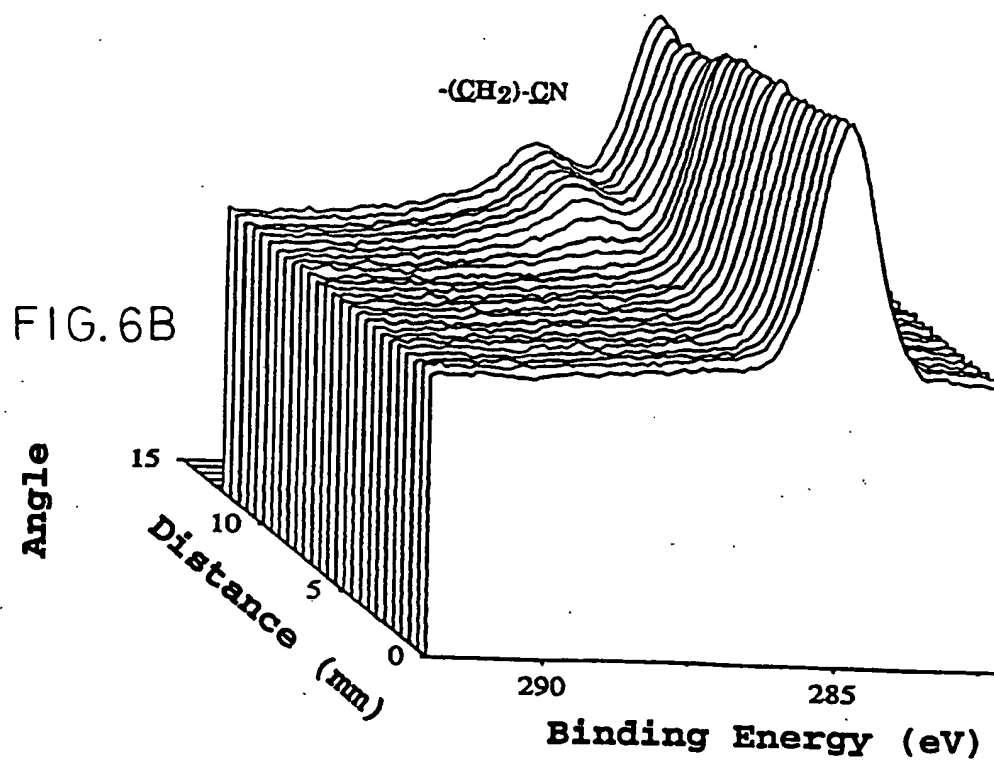


FIG. 6B

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 95/00736

## A. CLASSIFICATION OF SUBJECT MATTER

IPC6: G01N 33/558, G01N 33/543, G01N 33/553, G01N 33/545  
According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS, PASCAL, INSPEC

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SCIENCE, Volume 256, June 1992, Manoj K. Chaudhury et al, "How to Make Water Run Uphill" page 1539 - page 1541  -----  --	1-32

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

\* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&amp;" document member of the same patent family

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